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FURTHER STUDIES ON THE ADJUVANT FOR DRIED BCG VACCINE *

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SYNOPSIS

The authors describe some recent investigations undertaken in Japan with the object of finding an adjuvant for dried BCG vaccine which would make it possible for the vaccine to be stored at room temperature in tropical countries without suffering any serious loss of viability.

The first series of experiments, carried out with sodium glutamate as adjuvant, was designed to determine the effect of the concentration of adjuvant, the concentration of bacilli, the pH of the bacterial suspension, and the method of freeze-drying on the preservability of the vaccine. The sucrose vaccine in current use in Japan served as a control. In the light of the results, the authors make certain suggestions as to the optimum conditions for freeze-drying, but they stress that their proposals are tentative and that further research on the subject is needed.

In the second series of experiments the authors investigated the adjuvant properties of certain organic acids known to be involved in the metabolic reactions of the tubercle bacillus. Reasonably good results were obtained with α-ketoglutaric, malic, lactic, and citric acids, but none of these substances proved to be as satisfactory an adjuvant as sodium glutamate for storage at 37°C.

Studies carried out over the past few years have shown that dried BCG vaccine prepared with sodium glutamate as adjuvant can withstand greater thermal stress than the dried sucrose vaccine at present used in Japan. The ability of the glutamate vaccine to remain stable during storage at a temperature as high as 37°C was first detected by means of cultivation tests [1] and was later confirmed by immunization experiments in guinea-pigs [2] and by inoculation of human subjects.[a] In the last-mentioned report, it was stated that the allergenic potency of the glutamate vaccine stored at 37°C for one month was as strong as that of the ordinary sucrose vaccine after storage at 5°C for several months.

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[a] See paper by Obayashi et al. on page 275 of this number of the *Bulletin*.

Although these results strongly indicate that the glutamate vaccine is suitable for use under tropical conditions, there are still several important points to be studied. For example, not enough work has been done yet to determine either the optimum concentration of sodium glutamate or the best method of lyophilization for this particular adjuvant. Furthermore, very little is known about the mechanism by which an adjuvant exerts its protective effect on dried BCG, and it is hoped that research on this subject will lead to the discovery of more effective adjuvants.

In the present paper, some recent investigations undertaken with a view to settling some of the questions mentioned above are described.

Materials and Methods

Preparation of dried vaccine

Bacillary suspensions were prepared from 8- to 10-day-old second-generation Sauton (S_2) cultures of BCG, were diluted to the required concentration with adjuvant solution, and were dispensed into ampoules in 0.5-ml quantities.

Lyophilization was usually carried out by the method used in the routine production of dried vaccine: [6(a)] initial freezing at −15°C to −20°C for 1 hour; 7 to 8 hours' desiccation at a vacuum of 0.02-0.06 mm Hg, heating of the product being started 1 hour after the commencement of desiccation; for the last 3 hours, heating at approximately 30°C; etc.

Culture tests

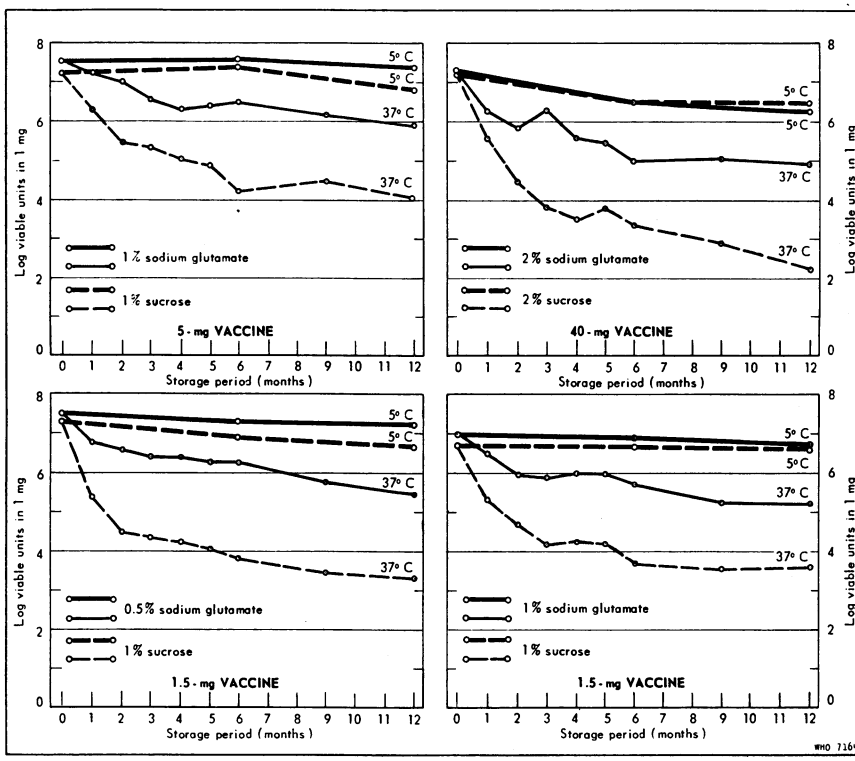
The dried vaccines prepared as described above were stored at 5°C and 37°C for various periods and were then submitted to culture tests. The contents of the ampoules were reconstituted with sterilized distilled water and subjected to serial tenfold dilutions to obtain the required bacterial concentrations; 0.1 ml of each of these dilutions was then inoculated on Ogawa's egg medium. For each dilution 5 slants were used. The colonies were counted after the slants had been incubated at 37°C for 4 weeks. As a general rule, 2 ampoules were used for each test and the results of the tests were expressed in numbers of viable units in 1 mg of bacillary mass.

Comparison of Preservability of Dried Glutamate Vaccine and Dried Sucrose Vaccine

Dried glutamate and sucrose vaccines prepared with various concentrations of bacilli and adjuvant were stored for 12 months both at 5°C and at 37°C, culture tests being carried out at intervals during this period.

The results of the culture tests are shown in Fig. 1.[a] No obvious difference was observed between the viable counts of the two kinds of vaccine after storage in the refrigerator, but a marked difference was apparent even after only 1 month's storage at 37°C, the decrease in viability of the glutamate vaccine being far smaller than that of the sucrose one at all concentrations of bacilli and adjuvant. This difference reached a maximum after storage for 2-3 months, and remained practically constant thereafter.

FIG. 1. COMPARISON OF PRESERVABILITY OF DRIED GLUTAMATE AND SUCROSE VACCINES AFTER STORAGE AT 5°C AND AT 37°C



As to the effect of the concentrations of bacilli and adjuvant on the stability of the resulting dried vaccine, no definite conclusions were reached in this experiment. This question will be discussed in greater detail in the next experiments.

[a] Further details of the results of these and other culture tests described in this paper are given in a set of tables and figures which have been deposited in the WHO Library ; photostat copies of this additional material may be obtained on request.

Relation between Concentration of Bacilli, Concentration of Sodium Glutamate, and Method of Lyophilization

With regard to the optimal concentration of sodium glutamate as adjuvant for dried BCG, Miller & Goodner [5] claimed that there was a definite optimum range between 0.1 % and 0.25 %, and that higher concentrations of adjuvant resulted in poorer survival; on the other hand, Cho & Obayashi [1] reported that, in their experiments with concentrations of 1 %, 5 % and 10 %, the best survival was obtained with the 1 % solution. However, as the latter authors point out, this matter will have to be considered in relation to the method of lyophilization and also to the concentration of bacilli.

Experiment 1

It had previously been observed by Cho that when glucose or sucrose was used as adjuvant and drying was carried out under the same mechanical conditions and in the same drying-chamber, the rate of evaporation decreased as the concentration of the adjuvant increased.[6 (b)] It was not known whether the same relation existed in the case of sodium glutamate, and the following experiment was therefore made.

Suspensions containing 10 mg of BCG per ml were prepared with distilled water and with 1 %, 2 % and 5 % solutions of sodium glutamate, and were dispensed into weighing-bottles in 2-ml quantities. For the controls, the same quantities of the solutions without bacilli were used. The materials, after being weighed, were subjected to the routine process of lyophilization. After they had been dried for the required period of time—4 hours for one half of the materials and 8 hours for the other—they were weighed again. The sublimation rate was calculated from the following formula: [a]

$$\frac{\text{weight of water vapour sublimated } [b]}{\text{weight of the material before drying}} \times 100$$

As shown in Table I, the rate of sublimation decreased as the concentration of sodium glutamate increased. Also, the rate of sublimation seemed to be a little greater when only the adjuvant solution was freeze-dried than when the adjuvant solution plus the bacillary suspension was treated. The latter finding is not in agreement with the results obtained earlier with glucose and sucrose, and further studies will have to be undertaken on this subject.

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[a] In our earlier report,[6(b)] we expressed our results in terms of the percentage of residual moisture:

$$\frac{\text{weight of vaccine after drying}}{\text{weight of vaccine before drying}} \times 100$$

Subsequently, we decided it would be better to use the sublimation rate, as defined in the present paper. We wish to express our sincere gratitude to Dr E. W. Flosdorf, Director, Research and Development Division, F. J. Stokes Corporation, USA, for his valuable suggestion in this connexion.

[b] The weight of water vapour sublimated is the difference between the weight of the material before drying and its weight after drying.

TABLE I. EFFECT OF CONCENTRATION OF SODIUM GLUTAMATE ON RATE OF SUBLIMATION

Type of adjuvant		Sublimation rate (%)	
		after 4 hours	after 8 hours
Distilled water	without bacilli	99.86	99.85
	with bacilli	99.24	99.53
1% sodium glutamate	without bacilli	98.53	98.68
	with bacilli	98.28	98.52
2% sodium glutamate	without bacilli	97.36	97.49
	with bacilli	97.31	97.36
5% sodium glutamate	without bacilli	94.21	94.18
	with bacilli	93.92	93.90

Experiment 2

10 mg per ml and 1 mg per ml suspensions of BCG were prepared with 1% sucrose and with 0.1%, 0.25%, 0.5%, 1% and 2% sodium glutamate as adjuvant and were dispensed into ampoules in 0.5-ml quantities. The suspensions were then subjected to the routine process of lyophilization. After 8 hours' desiccation, half of the ampoules were taken out and sealed, and the remainder were subjected to an additional 5 hours' desiccation at 40°C. The resulting dried vaccines were stored at 5°C and at 37°C for 12 months, culture tests being carried out several times during this period.

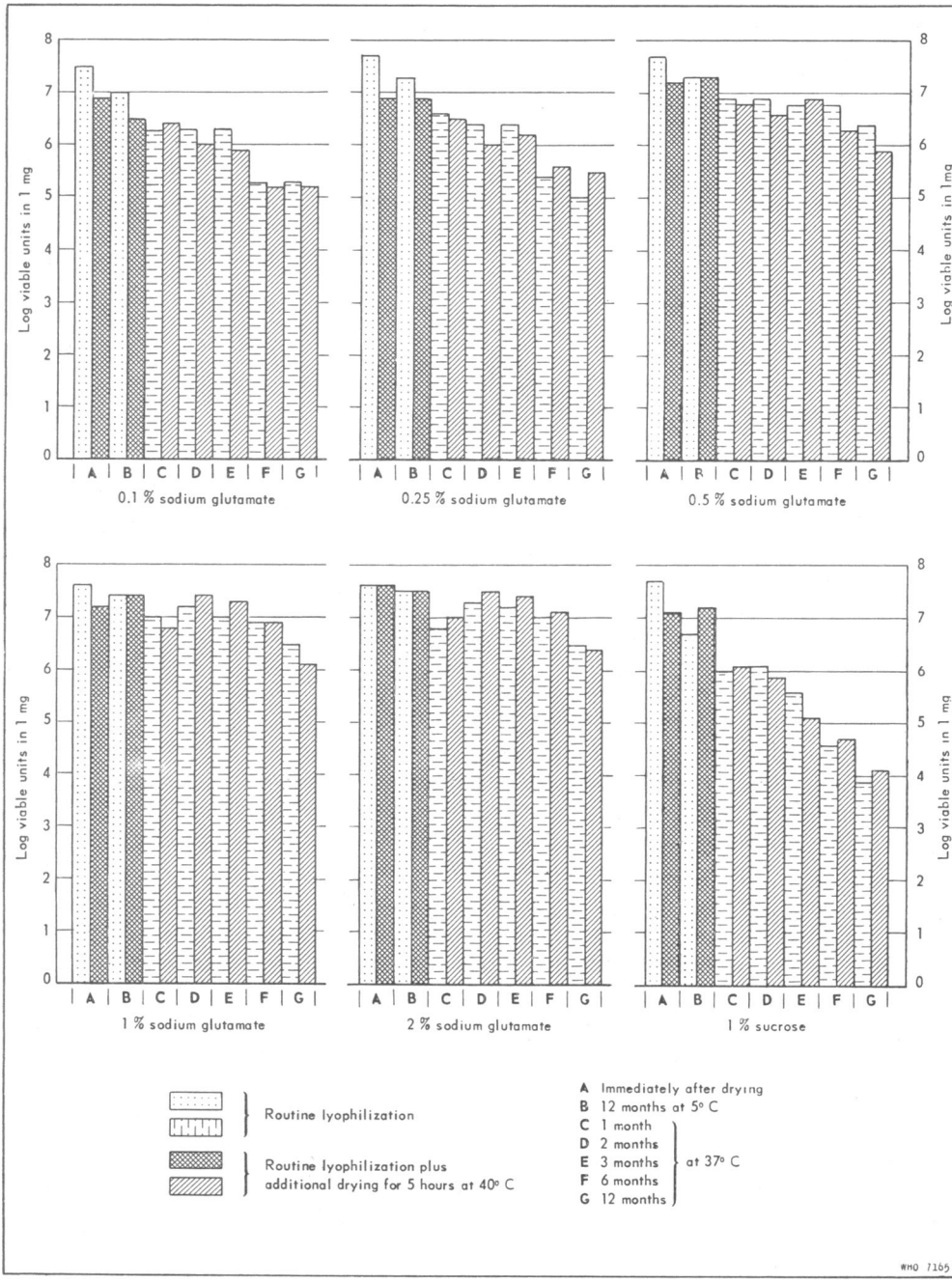
The results are shown in Fig. 2 and 3.[a] When the vaccines were stored at 5°C no significant difference in viability was observed among them. Thus, in this case, differences in the kind and concentration of adjuvant, in the amount of bacilli or in the method of freeze-drying did not have any perceptible influence on the preservability of the vaccine. On the other hand, when the vaccines were stored at 37°C, marked differences in viability were observed among them. These results can be summarized as follows:

1. *5-mg glutamate vaccine* (Fig. 2):

(*a*) In the range 0.1% to 0.5%, the stored vaccine revealed higher viability as the concentration of sodium glutamate increased; whereas in

[a] See footnote on page 257.

FIG. 2. EFFECT OF CONCENTRATION OF ADJUVANT AND METHOD OF LYOPHILIZATION ON PRESERVABILITY OF 5-mg DRIED VACCINE



the range 0.5% to 2%, no marked difference in preservability was observed with an increase in concentration.

(*b*) The effect of the difference in the drying method on the preservability of the vaccine was not marked, but in the case of the 1% and 2% adjuvant solutions, the additional drying seemed to have slightly increased the viability of the vaccine after preservation.

2. *0.5-mg glutamate vaccine* (Fig. 3):

(*a*) The same trend was observed in this vaccine as in the 5-mg glutamate vaccine; that is, between 0.1% and 0.5% (in the case of additional drying, 1%), the viability of the stored vaccine increased with increasing concentration of adjuvant.

(*b*) When the concentration of sodium glutamate was increased to 2%, a remarkable change was observed in the preservability. The number of viable units was zero from the third month onwards in the case of the vaccine dried by the routine method, and from the sixth month onwards in the case of the vaccine which had been subjected to additional desiccation.

(*c*) On the whole, the more thoroughly dried vaccine revealed a greater preservability than the one dried by the routine method. This difference was most obvious when 1% and 2% adjuvant solutions were employed.

3. *Effect of amount of bacilli on preservability* :

When the above data were further examined to find out the effect, on the preservability of the vaccines, of the difference in the amount of bacilli present, the following results were obtained:

(*a*) With adjuvant concentrations of 0.1% and 0.25%, the preservability was greater with the 0.5-mg vaccine than with the 5-mg one for both drying methods.

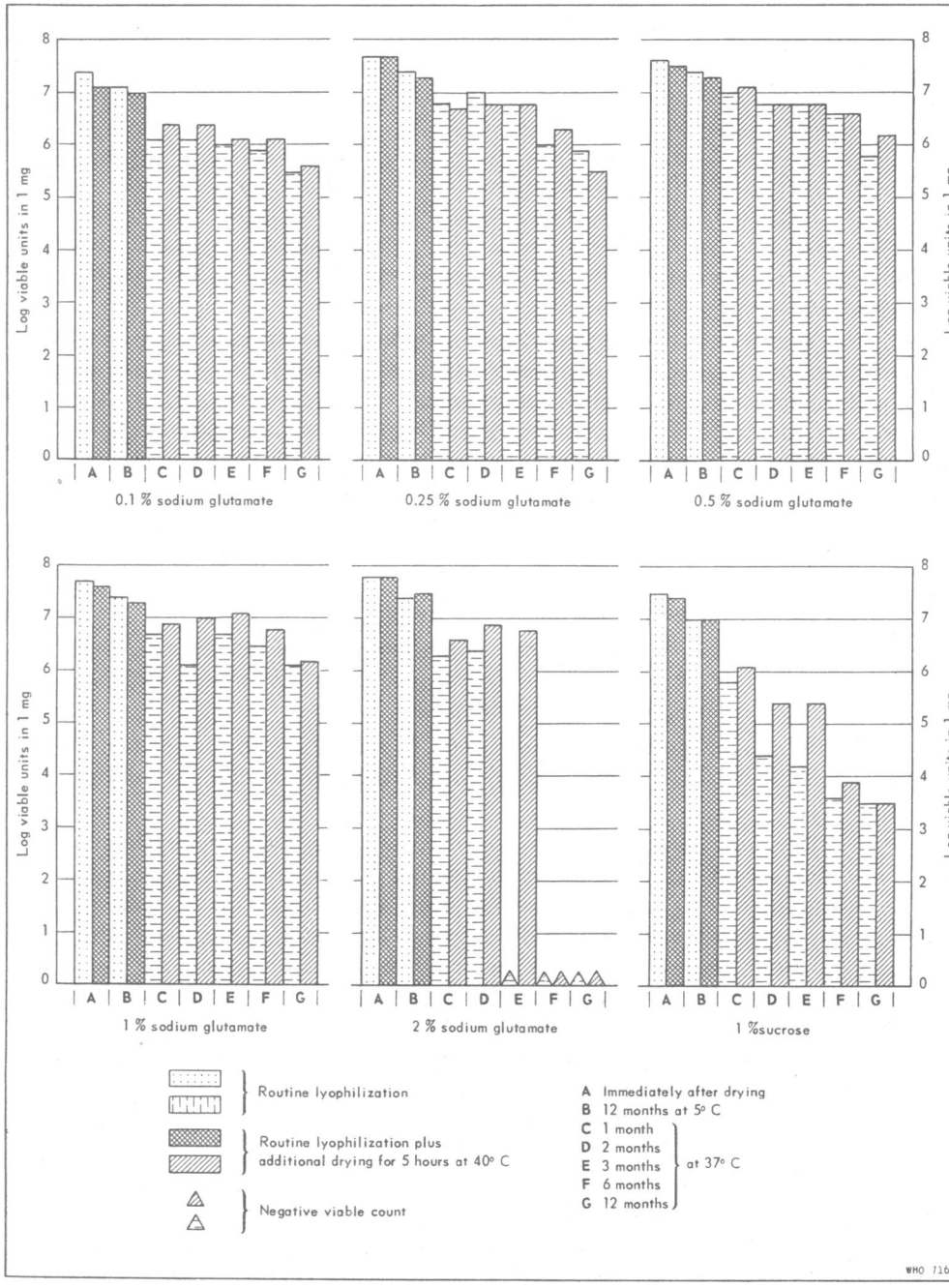
(*b*) When the concentration of adjuvant was 0.5%, there was no significant difference in preservability between the 0.5-mg and 5-mg vaccines, the similarity being particularly marked when the routine method of drying was employed; when additional drying was carried out, the preservability of the 0.5-mg vaccine was a little greater than that of the 5-mg one.

(*c*) With the 1% and 2% adjuvant solutions, the preservability of the 5-mg vaccine was greater than that of the 0.5-mg vaccine. The difference was especially marked in the case of the 2% solution; as mentioned earlier, the number of viable units in the 0.5-mg vaccine prepared with 2% adjuvant had fallen to zero by the third month (routine drying) and by the sixth month (additional drying).

4. *1% sucrose vaccine* :

(*a*) The decrease in viability of the dried sucrose vaccine during storage was more marked than that of the dried glutamate vaccines, except in the

FIG. 3. EFFECT OF CONCENTRATION OF ADJUVANT AND METHOD OF LYOPHILIZATION ON PRESERVABILITY OF 0.5-mg DRIED VACCINE



above-mentioned case of the 0.5-mg vaccine prepared with 2% sodium glutamate.

(*b*) On the whole, the 5-mg vaccine revealed a greater viability than the 0.5-mg vaccine.

(*c*) In the case of the 0.5-mg vaccine, the decrease in viability during storage was smaller with additional drying than with routine drying.

Experiment 3

In Experiment 2, concentrations of sodium glutamate up to 2% were tested, and, in the case of the 5-mg vaccine, 1% or 2% was considered a desirable concentration. Whether a further increase in concentration would or would not enhance the preservability was the next question to be settled.

10 mg per ml suspensions of BCG were prepared with 0.1%, 0.2%, 0.3%, 0.5%, 1%, 2%, 3%, 5% and 8% sodium glutamate, and were dispensed into ampoules in 0.5-ml quantities. In addition, 80 mg per ml suspensions were prepared from the same original suspension with 1% and 8% sodium glutamate, and were similarly dispensed into ampoules.

The freeze-drying of these vaccines was carried out in the following ways:

(*a*) Routine lyophilization procedure for 8 hours (heating at 20°C for the last 5 hours);

(*b*) 45 hours' desiccation at a comparatively low temperature, as follows: [a]

−20°C to −10°C for 20 hours;
−10°C to 0°C for 20 hours;
0°C to 10°C for 4 hours;
20°C for the last hour.

The resultant dried vaccines were stored at 5°C and at 37°C for 12 months, culture tests as usual being carried out several times during this period.

The results are shown in Fig. 4.[b] As in Experiment 2, no obvious difference in viability was observed when the vaccines were stored at 5°C, but after storage at 37°C a marked difference in the viable counts was apparent.

1. *5-mg vaccine dried at a low temperature* :

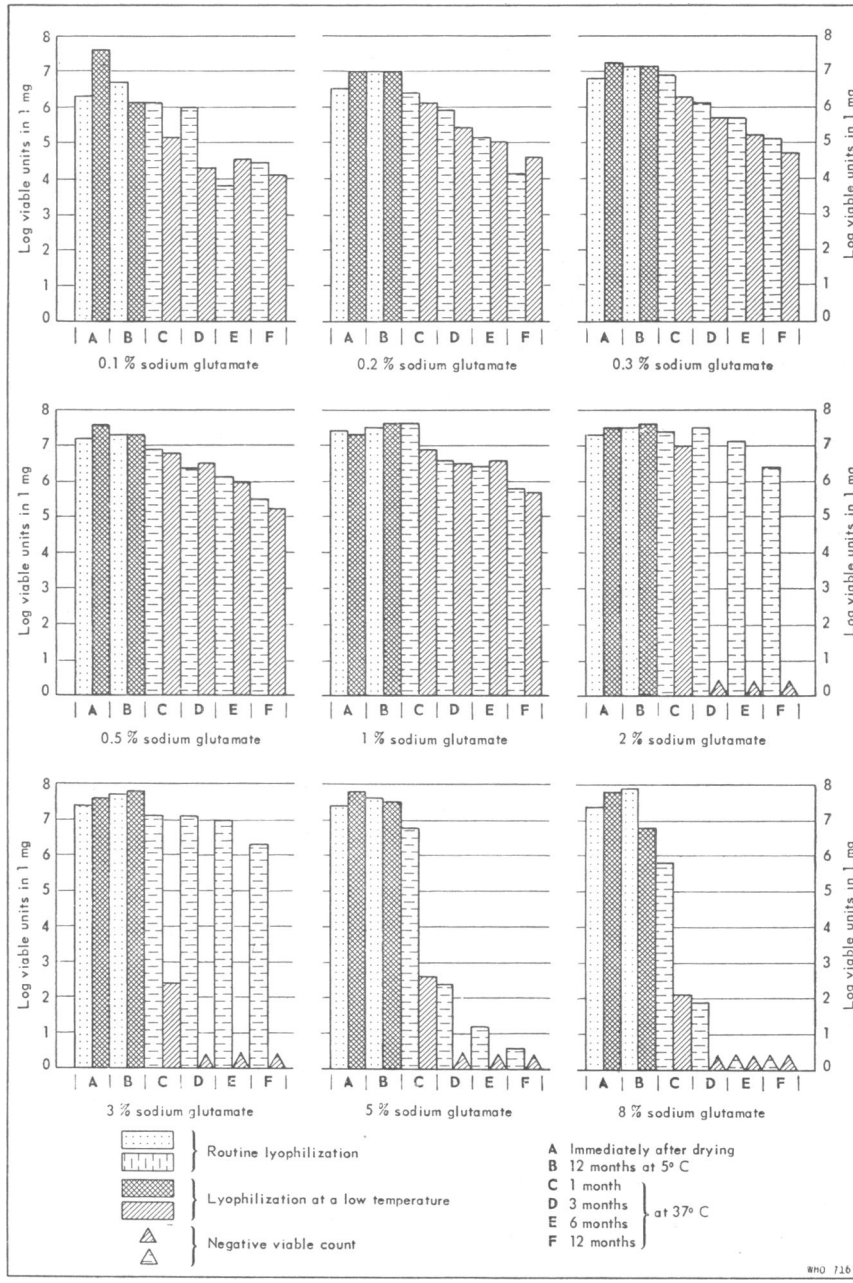
(*a*) In the range 0.1% to 1%, the viability of the stored vaccine increased with increasing concentration of adjuvant.

(*b*) When the concentration was raised to 2%, the resultant vaccine revealed a marked drop in viability after 3 months' preservation. When the concentration was 3% or higher, an appreciable decrease in viability was observed after only 1 month's preservation.

[a] In our experience, the moisture content of vaccine dried under these conditions is generally higher than that of vaccine dried by the routine method.

[b] See footnote on page 257.

FIG. 4. EFFECT OF CONCENTRATION OF ADJUVANT AND METHOD OF LYOPHILIZATION ON PRESERVABILITY OF 5-mg DRIED VACCINE



2. *5-mg vaccine dried by the routine method* :

(*a*) In the range 0.1 % to 2 %, the viability of the stored vaccine increased as the concentration of adjuvant increased.

(*b*) When the concentration was raised to 5 % or higher, a marked decrease in the viability of the stored vaccine was observed from the third month.

3. *40-mg vaccine* : [a]

The viability of this vaccine after preservation was higher with the 1 % than with the 8 % adjuvant. Furthermore, when 8 % adjuvant was employed, the 5-mg vaccine revealed a more marked and rapid decrease in viability during storage than the 40-mg vaccine did.

Experiment 4

The following experiment was made in order to determine the optimal concentration of adjuvant for 40-mg vaccine.

The preparation of the bacterial suspension and the filling of the ampoules were done in the same way as in Experiment 3. 0.5 %, 1 %, 2 %, 3 % and 5 % solutions of sodium glutamate were employed. The freeze-drying was carried out by the routine method.

The results of culture tests of the resultant vaccines after preservation are presented in Table II. No definite relation between the preservability of the vaccine and the concentration of adjuvant was revealed; the 5 % sodium glutamate appeared to give the best survival, but further studies will have to be carried out to confirm this.

TABLE II. VIABILITY OF DRIED BCG VACCINE CONTAINING 40 mg OF BACILLI PER AMPOULE PREPARED WITH VARIOUS CONCENTRATIONS OF SODIUM GLUTAMATE

Concentration of sodium glutamate	Number of viable units in 1 mg					
	immediately after drying	after 12 months at 5°C	after 1 month at 37°C	after 3 months at 37°C	after 6 months at 37°C	after 12 months at 37°C
0.5 %	4.5×10^8	8.0×10^8	3.0×10^5	6.8×10^5	7.7×10^4	4.0×10^4
1.0 %	1.6×10^7	8.0×10^8	1.5×10^7	2.0×10^8	3.4×10^5	4.9×10^5
2.0 %	3.8×10^8	3.2×10^7	2.2×10^8	2.8×10^5	4.4×10^5	1.9×10^5
3.0 %	1.6×10^7	2.8×10^7	8.4×10^8	1.0×10^8	4.5×10^5	2.3×10^5
5.0 %	1.8×10^7	5.3×10^7	1.4×10^7	5.0×10^8	2.9×10^8	9.0×10^5

[a] The results for this vaccine are not shown in Fig. 4, but are given in one of the tables deposited in the WHO Library.

It was noted that the solubility of the 40-mg vaccine became very poor when the concentration of adjuvant decreased; this might account for the inconclusive results of the culture tests.

Effect of Hydrogen Ion Concentration on Preservability of Dried BCG Vaccine

Studies were made in order to find out whether the pH of the sodium glutamate solution would have an effect on the preservability of the dried vaccine. 2% glutamic acid solutions with different pH values, such as 5.0, 6.0, 7.0, 8.0 and 9.0, were prepared by addition of NaOH, and sterilized. With these solutions, 10 mg per ml BCG suspensions were prepared, and were dispensed into ampoules in 0.5-ml quantities. The suspensions were freeze-dried by the routine method, and the dried vaccines were stored and tested for preservability as usual.

The pH was measured colorimetrically. After sterilization, the reaction was found to be more acid, as shown in Table III. It was also found that the best survival of bacilli was obtained when the reaction was approximately neutral.

TABLE III. EFFECT OF HYDROGEN ION CONCENTRATION ON PRESERVABILITY OF DRIED BCG VACCINE PREPARED WITH SODIUM GLUTAMATE

pH		Number of viable units in 1 mg		
before sterilization	after sterilization	immediately after drying	after 1 month at 37°C	after 2 months at 37°C
5.0	4.3	3.3×10^6	3.7×10^6	1.0×10^5
6.0	4.7	2.0×10^7	1.2×10^7	4.0×10^6
7.0	6.2	2.0×10^7	2.2×10^7	7.8×10^6
8.0	7.1	2.2×10^7	1.6×10^7	8.5×10^6
9.0	8.8	2.3×10^7	1.4×10^7	5.8×10^6

Effect of Addition of Sucrose to Sodium Glutamate on Preservability of Dried BCG Vaccine

Though sugar exerts a protective effect on dried BCG vaccine when the latter is stored at refrigerator temperature, it does not do so during storage at a high temperature; it may even be possible that, in the latter case, it has a definitely adverse effect on the survival of bacilli. On the

other hand, sodium glutamate has a favourable effect on survival, whether the vaccine is stored at refrigerator temperature or at incubator temperature.

In view of these facts, experiments were planned to determine the combined effect of these two adjuvants on the preservability of dried BCG vaccine.

Four kinds of adjuvant solution were prepared: 1% sucrose; 1% sodium glutamate; a mixture containing 1% of both sucrose and sodium glutamate; and a mixture containing 0.5% of each of the above substances. It was decided to include the latter mixture in view of the possibility that sublimation might proceed more slowly when a solution containing 1% of both sucrose and sodium glutamate is used as adjuvant than when either 1% sucrose or 1% sodium glutamate is used separately; it was felt that, if the concentration of each constituent were reduced to 0.5%, the rate of sublimation of the suspension might approximate more closely to that of the suspensions prepared with the individual 1% adjuvants.

With these adjuvants, 5 mg per ml suspensions of BCG were prepared and dispensed into ampoules in 0.5-ml quantities. The suspensions were freeze-dried by the routine method, but with 10 hours' desiccation.

The results of the culture tests of the resultant dried vaccines are shown in Table IV. After storage at 37°C the best survival was obtained with 1% sodium glutamate; after this came, in decreasing order, 1% sodium glutamate plus 1% sucrose, 0.5% sodium glutamate plus 0.5% sucrose, and 1% sucrose. Thus, during preservation of the vaccine at incubator temperature, the presence of sucrose was found to impair the adjuvant action of sodium glutamate.

TABLE IV. EFFECT OF ADDITION OF SUCROSE TO SODIUM GLUTAMATE ON PRESERVABILITY OF DRIED BCG VACCINE

Type of adjuvant	Number of viable units in 1 mg					
	immediately after drying	after 12 months at 5°C	after 1 month at 37°C	after 3 months at 37°C	after 6 months at 37°C	after 12 months at 37°C
1% sodium glutamate	2.8×10^7	3.1×10^7	1.7×10^7	6.4×10^6	5.3×10^6	1.4×10^6
1% sodium glutamate + 1% sucrose	2.8×10^7	5.6×10^7	1.3×10^7	4.4×10^6	3.4×10^6	7.0×10^3
0.5% sodium glutamate +0.5% sucrose	2.3×10^7	3.1×10^7	6.7×10^6	7.4×10^5	5.0×10^4	5.0×10^3
1% sucrose	1.4×10^7	4.7×10^6	2.0×10^5	3.8×10^5	8.4×10^3	2.7×10^3

TABLE V. EFFECT OF ORGANIC ACIDS ON PRESERVABILITY OF DRIED BCG VACCINE: EXPERIMENT 1

Type and concentration of adjuvant	pH adjustment with	pH after sterilization	Number of viable units in 1 mg					
			immediately after drying	after 6 months at 5°C	after 1 month at 37°C	after 2 months at 37°C	after 3 months at 37°C	after 6 months at 37°C
1% malic acid	NaOH	6.4	2.8×10^7	8.2×10^6	3.3×10^6	1.3×10^6	4.5×10^5	8.5×10^4
	NH_4OH	6.3	1.0×10^7	9.8×10^6	1.8×10^4	6.2×10^2	0 (10^{-1})	0 (10^{-1})
2% malic acid	NaOH	6.6	2.0×10^7	9.5×10^6	1.5×10^5	1.1×10^5	2.0×10^4	3.0×10^2
	NH_4OH	6.4	7.3×10^6	6.8×10^6	1.0×10^5	1.5×10^3	0 (10^{-1})	0 (10^{-1})
1% lactic acid	NaOH	4.8	1.0×10^6	6.0×10^5	4.0×10^5	6.0×10^4	2.0×10^4	1.0×10^4
	NH_4OH	4.5	5.0×10^1	2.9×10^4	0 (10^{-2})	0 (10^{-1})	0 (10^{-1})	0 (10^{-1})
2% lactic acid	NaOH	4.8	2.8×10^6	1.1×10^6	3.3×10^5	7.7×10^4	2.5×10^4	1.3×10^4
	NH_4OH	4.4	2.3×10^5	3.0×10^4	0 (10^{-2})	0 (10^{-1})	0 (10^{-1})	0 (10^{-1})
1% succinic acid	NaOH	6.6	3.0×10^5	1.0×10^5	1.0×10^4	5.8×10^3	5.3×10^2	2.0×10^2
	NH_4OH	6.5	3.0×10^5	2.2×10^5	7.8×10^4	1.3×10^4	6.3×10^3	1.0×10^3
2% succinic acid	NaOH	6.8	1.0×10^6	1.2×10^6	0 (10^{-4})	3.8×10^2	1.3×10^2	5.2×10
	NH_4OH	6.6	1.5×10^6	7.0×10^5	2.0×10^5	3.5×10^4	2.5×10^4	1.5×10^3
1% sodium glutamate		6.2	1.5×10^7	1.0×10^7	3.0×10^6	2.2×10^6	7.8×10^5	1.7×10^5
1% sucrose		5.4	1.3×10^6	3.2×10^5	1.5×10^4	4.5×10^3	1.0×10^3	2.1×10^2

The figures in parentheses indicate the amount (mg) of inoculum in the test in question.

Organic Acids as Adjuvants for Dried BCG Vaccine

The fact that sodium glutamate is effective in preserving dried BCG vaccine at 37°C has given us a clue to the significance of the adjuvant in the freeze-drying process. Glutamic acid is used as a source of nitrogen in synthetic media for the culture of tubercle bacilli, being easily utilized by the latter—a fact which suggests that the adjuvant may serve as a nutrient for the bacilli, especially when the dried vaccine is stored at a temperature as high as 37°C. However, since it will be very difficult to obtain direct proof of this supposition, we decided to approach the problem in another way—namely, by testing the adjuvant action of several substances which are considered to act as metabolites for the tubercle bacillus. In this study

we used a number of organic acids—malic, lactic, succinic, citric, pyruvic, α-ketoglutaric and oxaloacetic—which are thought to play a role in the series of bacterial metabolic reactions commonly known as the " Krebs tricarboxylic acid cycle ".

Experiment 1

In the first experiment, both 1% and 2% solutions of malic, lactic and succinic acids were used. The pH was adjusted to 7.0 both with NaOH and with NH_4OH, but in each case the reaction was found to be on the acid side after sterilization. As controls, 1% solutions of sodium glutamate and sucrose were employed.

A 5-mg vaccine was prepared as usual, freeze-dried by the routine method, stored at 5°C and at 37°C, and submitted to cultivation tests.

The results are presented in Table V. The best survival was obtained with sodium glutamate, and the next best with 1% malic acid adjusted with NaOH. In general, the use of NaOH for adjustment of the pH gave better results than the use of NH_4OH. No obvious difference in survival was observed between the 1% and 2% adjuvant solutions.

Experiment 2

1% and 0.5% solutions of pyruvic, citric, α-ketoglutaric and oxaloacetic acids were employed. NaOH was used for adjustment of the pH. The experimental procedure was the same as in Experiment 1.

As shown in Table VI, there was no obvious difference in the viability of the dried vaccines after storage in the refrigerator, but a marked difference was apparent after storage at 37°C. The best survival was obtained with the sodium glutamate and α-ketoglutaric acid vaccines, which revealed similar degrees of viability. The other vaccines, including the control vaccine with sucrose, showed far inferior preservability.

Experiment 3

1% solutions of malic, citric, α-ketoglutaric, lactic, pyruvic, and oxaloacetic acids were used. The pH of these adjuvants was adjusted with NaOH before sterilization in such a way as to ensure that the reaction of the bacterial suspensions would be neutral or weakly acid. The freeze-drying and testing were carried out in the same manner as in the preceding experiments.

As shown in Table VII, the best survival was obtained with sodium glutamate. Four of the organic acids (α-ketoglutaric, malic, lactic and citric) gave fairly good results, but pyruvic acid, oxaloacetic acid and sucrose yielded vaccines with inferior preservability. The result with lactic acid was not in agreement with that obtained in Experiment 1, where only a

TABLE VI. EFFECT OF ORGANIC ACIDS ON PRESERVABILITY OF DRIED BCG VACCINE: EXPERIMENT 2

Type and concentration of adjuvant	Final pH of bacterial suspension	Number of viable units in 1 mg					
		immediately after drying	after 7 months at 5°C	after 1 month at 37°C	after 2 months at 37°C	after 3 months at 37°C	after 7 months at 37°C
1% pyruvic acid	5.8	1.8×10^7	9.2×10^6	4.0×10^5	5.2×10^4	1.6×10^4	5.0×10
0.5% pyruvic acid	5.6	1.3×10^7	8.0×10^5	1.3×10^5	1.0×10^4	5.0×10^2	2.0×10
1% citric acid	6.6	2.3×10^7	7.5×10^6	1.2×10^6	2.5×10^5	4.0×10^4	4.5×10^2
0.5% citric acid	6.7	1.0×10^7	2.5×10^6	2.0×10^5	2.0×10^4	5.8×10^3	2.2×10^2
1% α-ketoglutaric acid	6.2	1.5×10^7	1.4×10^7	4.8×10^6	5.8×10^6	2.6×10^6	3.0×10^5
0.5% α-ketoglutaric acid	6.2	1.6×10^7	3.5×10^6	3.6×10^6	8.4×10^5	2.2×10^6	2.8×10^5
1% oxaloacetic acid	8.8	1.1×10^7	1.5×10^6	1.0×10^5	2.3×10^3	1.3×10^4	3.2×10^2
0.5% oxaloacetic acid	8.6	1.3×10^7	1.5×10^6	3.3×10^6	2.9×10^4	3.0×10^3	8.0×10
1% sodium glutamate	6.0	2.0×10^7	7.0×10^6	5.3×10^6	3.5×10^6	1.6×10^6	4.2×10^5
1% sucrose	5.6	1.5×10^6	1.0×10^6	6.8×10^4	7.0×10^4	8.3×10^4	5.0×10^3

TABLE VII. EFFECT OF ORGANIC ACIDS ON PRESERVABILITY OF DRIED BCG VACCINE: EXPERIMENT 3

Type of adjuvant (1%)	Initial pH of adjuvant	Final pH of bacterial suspension	Number of viable units in 1 mg			
			immediately after drying	after 1 month at 37°C	after 2 months at 37°C	after 3 months at 37°C
Malic acid	7.3	6.3	1.4×10^7	6.0×10^6	3.0×10^6	2.7×10^6
Citric acid	7.2	6.6	6.5×10^6	2.8×10^6	1.3×10^6	9.8×10^5
α-ketoglutaric acid	7.2	6.2	7.3×10^6	3.0×10^6	2.2×10^6	1.0×10^6
Lactic acid	9.6	6.0	6.5×10^6	3.2×10^6	1.6×10^6	2.1×10^6
Pyruvic acid	6.9	6.2	6.5×10^6	1.5×10^6	2.2×10^5	7.0×10^4
Oxaloacetic acid	10.0	7.0	3.5×10^6	1.0×10^6	3.2×10^5	1.7×10^5
Sodium glutamate	7.0	6.0	1.3×10^7	1.3×10^7	4.2×10^6	2.0×10^7
Sucrose	7.0	5.8	1.0×10^7	7.2×10^5	5.5×10^5	3.1×10^4

poor survival was observed; this may be due to the fact that the reaction of the adjuvant in the latter experiment was strongly acid.

On the whole, the above experiments can be said to have proved that there is a group of organic acids which afford dried BCG vaccine a fairly good protection against thermal stress. Although the degree of survival after storage at 37°C fell a little short of that shown by dried sodium glutamate vaccine, it was far higher than that observed in dried sucrose vaccine.

Discussion

The experiments described in this paper have provided some useful information about the role of the adjuvant in dried BCG vaccine.

It has been found that, like glucose and sucrose, sodium glutamate ensures the retention of moisture in the vaccine, and that the rate of sublimation diminishes as the concentration of sodium glutamate increases—a relation which had been demonstrated earlier in the case of glucose and sucrose.

Furthermore, it has been observed that there is a concentration of adjuvant beyond which a further increase in concentration results in a marked decrease in the viability of the vaccine after storage, and that this limiting concentration can be raised by drying the vaccine more thoroughly.

It can be concluded from the above facts that there is an upper limit to the moisture content of dried BCG vaccine, and that an increase of moisture beyond this limit is definitely detrimental to the living organisms.

Whether or not there is also a lower limit to the moisture content of dried BCG vaccine is not such an easy question to answer. It has been observed in our experiments that the viability of the preserved vaccines prepared with comparatively low concentrations of adjuvant (0.1%-0.5%) increases as the concentration of adjuvant increases (Fig. 2, 3 and 4). This seems to point to the existence of an optimal degree of dryness for the preservation of living bacilli, beyond which further drying is unfavourable for survival. But other findings are not in agreement with this assumption: for example, no significant difference in preservability was found between the vaccine which was dried by the routine method and the vaccine which was more thoroughly dried, when comparatively low concentrations of adjuvant were employed. Further studies on this subject are needed.

The question of the concentration of bacilli must be considered in relation to the concentration of adjuvant and to the method of freeze-drying.

With adjuvant concentrations in the range 0.1% to 0.25%, the preservability after normal drying was greater in the case of the 0.5-mg vaccine than in that of the 5-mg vaccine, whereas with higher concentrations of

adjuvant the reverse was true, the 5-mg vaccine showing the greater viability. With 2% adjuvant, a marked decrease in the viability of the 0.5-mg vaccine was apparent after only 3 months' preservation. The results obtained with the more thoroughly dried vaccine were similar. In this case, the 0.5-mg vaccine revealed the greater viability up to a concentration of 0.5%, but thereafter showed poorer survival than the 5-mg vaccine. With 2% adjuvant, an obvious decrease in the viability of the 0.5-mg vaccine was observed from the sixth month onwards.

The above findings are easily explainable on the assumption that the content of residual moisture is greater in the 0.5 mg vaccine than in the 5-mg vaccine. From our experiment on the rate of sublimation, it can be concluded that the absolute amount of residual moisture may be a little larger in 5-mg vaccine than in 0.5-mg vaccine, but that the relative amount, that is, the amount of residual moisture per unit weight of bacillary mass, will be far larger in 0.5-mg vaccine than in 5-mg vaccine. For this reason, we shall probably have to carry out freeze-drying for a longer period in the case of 0.5-mg vaccine than in the case of 5-mg vaccine, especially when the concentration of adjuvant is relatively high.

As to the concentration of adjuvant and the conditions of freeze-drying to be recommended in the routine production of vaccine, the following suggestions are made in the light of our recent experiments.

When the amount of bacilli in one ampoule is 5 mg or so (0.5 ml of 10 mg per ml suspension), a comparatively high concentration of adjuvant, that is, 1% or 2%, will be desirable; and it will be better and safer to carry out a little more thorough drying. This additional drying will be particularly important when 2% adjuvant is used. In one of our experiments, good survival was obtained up to a concentration of 3% (Fig. 4), but an obvious decrease in the viability took place when the concentration was raised to 5% or higher, and we do not think it safe to use 3% sodium glutamate in the routine production of dried vaccine; it may, however, be possible to use this concentration if additional drying is carried out.

When the amount of bacilli in one ampoule is as small as 0.5 mg, an adjuvant concentration of 0.5% to 1% will be adequate, and it will be better to dry the vaccine a little more thoroughly than in our routine procedure.

As to 40-mg vaccine, sufficient results have not yet been obtained to enable us to recommend any definite method of production.

The fact that, even when the concentration of adjuvant is too high, or the period of freeze-drying is too short, and consequently the resulting vaccine is not sufficiently dry, there may be no marked decrease in viability when the vaccine is preserved at refrigerator temperature is of special importance, since it means that, in such a case, we can find out whether the concentration is suitable for the particular method of freeze-drying employed, simply by making culture tests of the vaccine after storage at incubator temperature.

The question of the unsuitability of sucrose as an adjuvant for vaccine which is stored at a high temperature is of great interest. In our experiment, it was found that the addition of sucrose to sodium glutamate, instead of enhancing the preservability, had a definitely adverse effect on survival. On the other hand, Fry & Greaves [4] obtained good survival in the freeze-drying of the paracolon bacillus by using as adjuvant the so-called " Mist. desiccans ", which is a mixture of one part of broth and three parts of serum with 7.5% glucose. These workers claimed that the presence of glucose ensured the retention of a certain amount of moisture, which was necessary for survival. In view of this, we consider that in our experiment, the addition of sucrose had an adverse effect on preservability not because it rendered the amount of residual moisture inadequate for survival, but because sucrose itself is harmful to the bacilli at a high temperature.

As to the effect of pH on the preservability of dried BCG, it was found that the best survival of bacilli was obtained when the reaction was approximately neutral. The effect of pH was not so marked, however, in our experiment as it was in the study by Miller & Goodner,[5] where phosphate buffer solution was used as adjuvant and even a slightly acid reaction (pH 6.5) was reported as being detrimental to survival. The relatively mild effect of pH in our experiment may be due to the protective effect of sodium glutamate itself. In this connexion, Dubos,[3] in his study on the effect of metabolites on the viability of the tubercle bacillus in an acid environment, reported that glutamic acid was one of the metabolites which, when added to culture media with an acid reaction, permitted microbial growth to take place.

Besides sodium glutamate, several substances which are fairly effective for the preservation of dried BCG at a high temperature have been found. These substances are organic acids, such as α-ketoglutaric, malic, lactic and citric acids, which are considered to be important in the metabolism of the tubercle bacillus. It is hoped that, by approaching the problem along these lines, it will be possible to find out more about the mechanism of adjuvant action.

RÉSUMÉ

Les expériences faites jusqu'à maintenant sur la résistance à la chaleur du BCG desséché ont montré la supériorité du vaccin au glutamate sur le vaccin au saccharose. Bien que le vaccin au glutamate paraisse réunir les conditions posées par la vaccination en pays tropical, divers points restent à préciser. Il s'agit en particulier de déterminer la concentration optimum du glutamate de sodium, la meilleure méthode de lyophilisation avec cet adjuvant, et d'élucider la nature de la protection exercée par le glutamate sur le vaccin.

Les auteurs ont étudié quelques-uns de ces problèmes. Ils ont constaté que le glutamate, comme le glucose et le saccharose, assure la rétention d'une certaine quantité d'humidité et que le taux de sublimation diminue à mesure qu'augmente la concentration de

l'adjuvant. Il existe cependant une concentration optimum, au-delà de laquelle la viabilité du vaccin diminue. En d'autres mots, il existe une limite supérieure d'humidité du BCG qui, si elle est dépassée, nuit au pouvoir de conservation du vaccin. Quant à la limite inférieure, elle n'a pas encore été déterminée.

Pratiquement, une concentration d'adjuvant de 1%-2% est à recommander pour une quantité de bacilles de 5 mg par ampoule; une proportion de 0,5%-1% convient à une concentration bacillaire de 0,5 mg par ampoule. Un pH voisin de la neutralité est particulièrement favorable, bien que ses variations ne paraissent guère influencer les qualités de conservation du vaccin, en raison peut-être du pouvoir protecteur qu'exerce le glutamate. Diverses autres substances contribuant à assurer la conservation du BCG à des températures élevées ont été mises à l'étude, telles que les acides α-cétoglutarique, lactique, malique et citrique, qui jouent un rôle dans le métabolisme du bacille tuberculeux.

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